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Review paper

Proteasome inhibitors as anti-cancer agents

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The ubiquitin (Ub)–proteasome pathway is the major non-lysosomal pathway of proteolysis in human cells and accounts for the degradation of most short-lived, misfolded or damaged proteins. This pathway is important in the regulation of a number of key biological regulatory mechanisms. Proteins are usually targeted for proteasome-mediated degradation by polyubiquitinylation, the covalent addition of multiple units of the 76 amino acid protein Ub, which are ligated to ε-amino groups of lysine residues in the substrate. Polyubiquitinylated proteins are degraded by the 26S proteasome, a large, ATP-dependent multicatalytic protease complex, which also regenerates monomeric Ub. The targets of this pathway include key regulators of cell proliferation and cell death. An alternative form of the proteasome, termed the immunoproteasome, also has important functions in the generation of peptides for presentation by MHC class I molecules. In recent years there has been a great deal of interest in the possibility that proteasome inhibitors, through elevation of the levels of proteasome targets, might prove useful as a novel class of anti-cancer drugs. Here we review the progress made to date in this area and highlight the potential advantages and weaknesses of this approach. [© 2000 Lippincott Williams & Wilkins.]

Key words: Inhibitor, proteasome, ubiquitin.

Proteasome structure and activities

20S (700 kDa) proteasomes are located both in the nucleus and in the cytoplasm,[1–5] and can constitute up to 1% of the soluble protein in cell extracts.[6] Each of these multicatalytic protease complexes consists of 28 subunits, 14 of each of two classes termed α and β. These subunits are arranged as four heptameric rings stacked in a pseudo-helical arrangement to form a hollow cylinder.[7] The outer rings consist of α subunits and the β subunits, which contribute the protease active sites, form the two inner rings. The central channel includes three cavities, the central one formed by the β subunits, which are arranged such that their catalytic sites are accessible only from this internal space. Narrow pores at the top and bottom of the hollow cylinder, which may be regulated by accessory complexes, are thought likely to provide the route by which unfolded substrate proteins gain access to the internal compartments (Figure 1).

The ability of the proteasome to perform multiple functions is due in part to its broad specificity for peptide bond cleavage. Several peptidase activities have been described in eukaryotic proteasomes; there are two trypsin-like activities, three chymotrypsin-like activities,[8] two peptidyl-glutamyl peptide-hydrolyzing activities,[8,9] a branched amino acid preferring and a small neutral amino acid preferring activity.[10] Cleavages performed by the proteasome are relatively non-specific, typically producing peptide fragments of 6–9 amino acid residues. The proteasome has been classified as a threonine protease, as the N-terminal threonine of the mature proteasome β subunit provides the nucleophile which attacks the carbonyl group of the peptide bond.[11,12]

A number of additional complexes that activate, inhibit or modulate proteasome function have been identified.[4,13,14] Among these, the two best studied examples are the 19S (PA700) regulatory complex of the 26S proteasome[4] and the 11S regulator (REG/PA28).[15] The 26S proteasome is a large (1500–2000 kDa) complex consisting of the catalytic core, the 20S proteasome, together with the 19S regulatory complex, which has no proteolytic activity of its own but confers on the complex the unique ability to degrade polyubiquitinylated proteins, as well as non-ubiquitinylated proteins. ATP is required both for the

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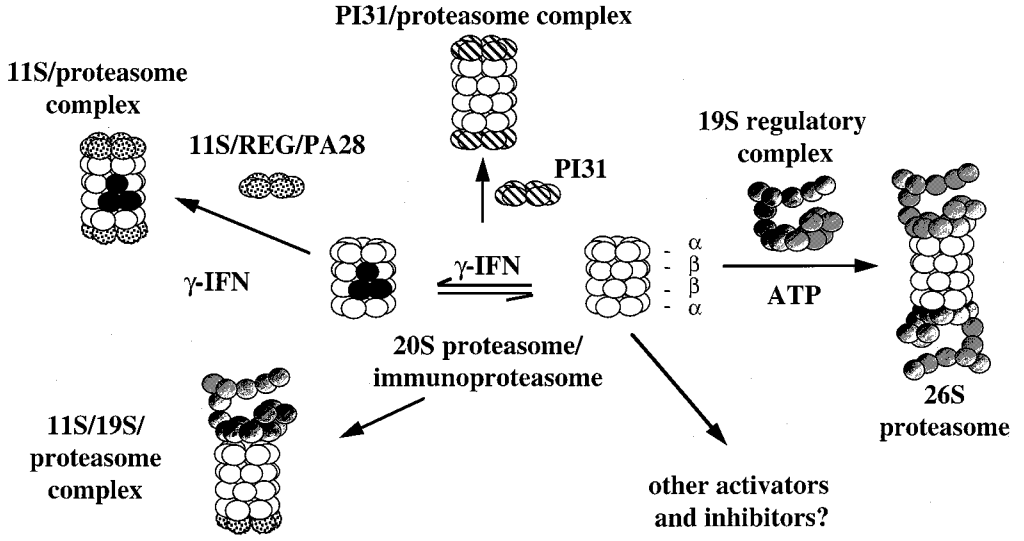

Figure 1. Alternative forms of the proteasome. The core 20S proteasome comprises stacked rings of α and β (catalytic) subunits. In response to IFN-γ, three of the β subunits are replaced by alternative subunits and the 20S proteasome is capped by the 11S/REG/PA28 complex. The PI31 complex can inhibit 20S proteasome activity, while the 19S regulatory complex confers ATP dependence and the ability to degrade polyubiquitinylated substrates. The potential exists for different combinations of modulators (such as the 11S and 19S regulators) to bind simultaneously to any given 20S complex. For details, see text.

formation of the 26S proteasome from these two complexes and for the degradation of substrates. The 19S regulatory complex consists of least 17 subunits,[16,17] which are thought to be involved in the recognition and binding of polyubiquitinylated proteins, unfolding of the substrate, translocation of the unfolded polypeptide chain to the proteasome and disassembly of the polyubiquitin chain. These subunits include six ATPases that have an ATP-dependent protein unfolding activity and form a ring-like structure at the interface with the 20S proteasome.[18] The 26S proteasome is involved in a wide variety of cellular processes and the majority of the regulatory subunits are essential for cell viability in the simple eukaryotic model organisms where this question has been addressed.[19,20]

Ubiquitinylation is a multi-step reaction that requires three classes of enzyme termed E1–3. E1 enzymes activate ubiquitin (Ub) monomers, while the E2 enzymes transfer Ub from E1 to the protein substrates, which are in many cases bound to E3 Ub–protein ligases.[1,3] A chain consisting of four or more Ub residues is required for proteolysis of the target to occur.[21] This reflects the fact that protein modification by ubiquitinylation does not function solely to target proteins for degradation. Mono-ubiquitinylation, for example, appears to be required for the internalization of a number of transmembrane growth factor receptors.[22] Ub ligation is carried out by a variety of structurally diverse E3 enzymes, and is frequently rate limiting and subject to a high degree of regulation.

Three of the 14 constitutively expressed subunits of the mammalian 20S proteasome can be replaced by distinct, closely related subunits in response to interferon (IFN)-γ.[23] These subunits compete with the constitutive subunits during assembly of the complex to form immunoproteasomes, which have been implicated in generating peptide antigens for presentation by MHC class I molecules.[24–27] The 11S regulator, which consists of two types of subunit forming a six-membered ring, is also induced by IFN-γ. The binding of the 11S regulator to immunoproteasomes changes the quantity and quality of the peptides produced to favor the generation of dominant T cell epitopes.[28] A significant fraction of all nascent protein is directed for immediate proteasome-dependent proteolysis before completion of translation.[29,30] This explains how antigenic peptides derived from apparently stable intracellular proteins can be loaded onto class I molecules.

Proteasome inhibitors

Several classes of proteasome inhibitor have so far been identified.[31] These include peptide aldehydes,

peptide vinyl sulfones, peptide boronic acids, glyoxals and α^1,β^1-epoxyketone-containing peptides. Proteasome inhibitors have been used to implicate the proteasome in a number of processes including apoptosis, differentiation, degradation of misfolded proteins and antigen presentation. Most of these agents act primarily on the chymotrypsin-like activity of the proteasome, which is thought to be the rate-limiting step in protein degradation. Unfortunately most of these inhibitors have also been found to inhibit cellular proteases other than the proteasome. For example, lactacystin shows relatively high specificity for the proteasome,[32] but can also inhibit cathepsin A[33] and tripeptidyl peptidase II.[34] Only one inhibitor, epoxomicin, has so far been reported to be completely specific for the proteasome.[35]

Proteasome inhibitors as anti-cancer drugs

Several studies have shown that proteasome inhibitors can induce apoptotic cell death in cancer cell lines at doses that are comparatively non-toxic to untransformed cells.[36,37] Furthermore, the anthracycline anti-cancer agent aclacinomycin A was found to inhibit the proteasome,[38] although other reports classified this drug as a topoisomerase inhibitor. There has therefore been a great deal of interest in the possibility that proteasome inhibitors might prove useful as novel cancer therapeutic agents. In this respect the single most important end-point is clearly anti-tumor activity combined with acceptable levels of general toxicity. Given the wealth of knowledge about proteasome structure and function, however, it is also reasonable to ask whether a more rational approach based on this biological and biochemical knowledge might be possible. Studies in this area have therefore taken two complementary approaches. In the first, proteasome inhibitors characterized *in vitro* have been found to have significant anti-tumor activity in animal models and these have in some cases been taken into clinical trials. The second approach involves investigation of the effects of inhibitors on steady-state levels of specific biological regulatory proteins in tumors or tumor cell lines. The objective in this case is either to explain observed anti-tumor activity in terms of up-regulation of a specific biological pathway or pathways, or to target such pathways specifically.

Results using human tumor xenograft models are promising. Treatment of mice bearing a prostate PC-3 tumor with weekly i.v. injections of a dipeptide boronic acid proteasome inhibitor, PS-341, resulted in a significant (60%) decrease in tumor burden after 4 weeks of treatment.[39] PS-341 was able to penetrate the tumor and inhibit proteasome activity within 1 h of administration. Direct intratumoral injection on four consecutive days caused a substantial (70%) reduction in tumor volume, with two of the five treated mice having no residual tumor burden 7 weeks later. Similar results were reported using the Lewis lung model.[40] PS-341 accumulated principally in the liver, kidney, prostate, spleen and gastrointestinal tract of treated animals, with no apparent penetration into the CNS, eye or testes and was toxic to the bone marrow colony-forming unit granulocyte macrophage.[39,40] In primates the limiting toxicity was gastrointestinal, with modest effects seen in the spleen and thymus where lymphocytic depletion was reported. On the basis of these results PS-341 is currently in phase I evaluation in patients with a variety of advanced cancers.

In a second line of investigation, the peptidyl aldehyde proteasome inhibitor Z-LLF-CHO was found to be up to 40-fold more effective in inducing apoptosis in transformed fibroblasts or lymphoblastoid cells, in comparison to their untransformed counterparts.[41] Z-LLF-CHO was subsequently found to be effective against a Burkitt's lymphoma xenograft in SCID mice. Significant induction of early tumor regression and delay in tumor progression were associated with increased tumor cell apoptosis, after administration of a single interscapular dose. The effective dose was well tolerated, with no significant toxicity being detected.

Aside from approaches exploiting their potent cytotoxic effects, proteasome inhibitors are potentially applicable to an entirely separate anti-cancer strategy, i.e. the development of novel tumor vaccines. Since the proteasome generates antigenic peptides, treatment with proteasome inhibitors results in cell surface expression of unoccupied MHC class I molecules, which can then be loaded *in vitro* with synthetic peptides corresponding to tumor-associated antigens. In one recent study an antigenic peptide derived from MUC-1 was modified to increase its affinity for class I molecules. Fibroblasts treated with a proteasome inhibitor and loaded with this peptide induced a cytotoxic T lymphocyte response that significantly delayed tumor progression in a xenograft model.[42] Pre-immunized mice were also protected against the development of lung metastases following surgical removal of the primary tumor. Proteasome-inhibited, peptide-loaded fibroblasts can therefore act as efficient antigen-presenting cells and may represent an effective form of tumor vaccine in some cases. The applicability of this approach in human subjects awaits further investigation.

Explaining the anti-tumor activity of proteasome inhibitors at the molecular level

In addition to the important role for immunoproteasomes in antigen presentation, the Ub–proteasome pathway plays both positive and negative roles in cell cycle regulation, apoptosis and the control of transcriptional responses to a variety of stresses. Substrates relevant to these processes include tumor suppressor proteins, proto-oncogene products, other transcription factors and cell cycle regulatory proteins.[1,43,44] Proteasome-dependent proteolysis has a further important function in the degradation of proteins that become misfolded in the endoplasmic reticulum (ER). How can the specific anti-tumor effects described above be explained in terms of inhibition of these functions?

Cell cycle regulation

Cell cycle progression is driven by the sequential activation of multiple cyclin-dependent protein kinases (CDKs), which are inactive as monomers but activated through heterodimerization with cyclin proteins.[45] Levels of the various CDKs do not change dramatically during the cell cycle, whereas cyclin abundance is usually cell cycle phase-specific. Regulation of CDK–cyclin activity is also achieved in part through association with inhibitory proteins such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$. Both of these inhibitors, as well as several cyclins, are degraded by the Ub–proteasome pathway.[46–49] As with many other proteasome substrates, the specificity for degradation of these cell cycle-related proteins is conferred principally by E3 Ub ligases. In terms of cell cycle regulation, the best understood E3 enzymes are of the APC/C (anaphase-promoting complex/cyclosome) and SCF (Skp1/cullin/F-box) types.[50,51] Each of these is a multi-protein complex existing in multiple forms, each with its distinct substrate specificity. The most extensively characterized cell cycle transition involving Ub-dependent proteolysis is the transition from mitosis to interphase. This is induced by the degradation of cyclin B, which associates with Cdc2, the CDK responsible for establishment of the mitotic state.[52] Cyclin B proteolysis is controlled by an APC/C Ub ligase containing a Fizzy/Cdc20 specificity-determining subunit.[53] APC/C-directed ubiquitinylation is also responsible for targeting other mitotic regulators for proteolysis, including proteins whose degradation is essential for sister chromatid separation at the onset of anaphase.[54] In this case the APC/C specificity determinant is the Cdh1 protein.

Elimination of excess $p27^{KIP1}$ during late G_1 phase is required for G_1 cyclin/CDK complex activation and progression into S phase of the cell cycle. For a variety of tumor types, increased $p27^{KIP1}$ degradation by the proteasome (with maintenance of normal levels of *KIP1* mRNA) has been found to be associated with poor prognosis.[55–58] Accumulation of CDK inhibitor proteins is frequently associated not only with cell cycle arrest, but with suppression or induction of cell death, onset of senescence or differentiation. While the underlying mechanisms are not currently known, it seems likely that proteins such as $p21^{WAF1}$ and $p27^{KIP1}$ play key roles in the coordination of cell cycle withdrawal with other aspects of cellular behaviour.

Unsurprisingly, given the requirement for Ub-dependent proteolysis in several major cell cycle processes, cycling cells in general seem to be particularly sensitive to the toxic effects of proteasome inhibition. Selectivity for actively cycling versus quiescent cells has been observed following proteasome inhibitor treatment of endothelial cells,[59] hematopoietic progenitor cells[60] and Rat-1 fibroblasts.[61] However, proteasome inhibitors induced apoptosis in non-proliferating PC12 cells that had been induced to differentiate by treatment with nerve growth factor.[61] Thus cell proliferation is one determinant of the cellular response to proteasome inhibition, but not the only one.

Apoptosis

Apoptotic cell death is fundamentally important in a wide variety of processes during normal development and is widely considered to be a major determinant of response to anti-cancer therapies.[62] Cysteine aspartyl proteases of the caspase family perform both regulatory and effector roles in apoptosis, and degrade a wide variety of intracellular proteins.[63] Upstream from the caspases, many pro-apoptotic signals converge on the mitochondria, which respond by releasing cytochrome *c*, a potent trigger of cell death. Members of the Bcl-2 family act in part by governing mitochondrial cytochrome *c* release. The p53 tumor suppressor can promote apoptosis by up-regulating transcription of *BAX*, a pro-apoptotic, *Bcl-2*-related gene, in response to DNA damage; BAX in turn stimulates mitochondria to release cytochrome *c*.

BAX, Bcl-2 and p53 are all targets of the Ub–proteasome pathway,[64–66] although some p53 degradation may also be calpain mediated.[67] Under normal circumstances the level of p53 protein is suppressed through its association with the Mdm-2/HDM2 E3 Ub ligase,[68] which itself is transcriptionally activated by p53, creating a negative feedback loop. Mdm-2/HDM2

also functions as a direct inhibitor of p53-mediated transcriptional activation and to promote the export of p53 from the nucleus into the cytoplasm. Nuclear export appears to be important for the efficient degradation of p53, although the reason for this is not yet apparent. In response to diverse stimuli such as genomic damage and hypoxia, p53 levels increase allowing the activation of its transcriptional targets, a subset of which, including *BAX*, can function to elicit apoptosis.[69] The response to p53 activation is cell type- and context-dependent, however, as other genes activated by p53, including *WAF1* and *14-3-3σ*, promote cell cycle arrest rather than apoptosis.[70,71] Activation of p53 is accompanied by a reduction in its degradation by the 26S proteasome. Tumor-associated p53 mutations frequently lead to loss of transcriptional activation activity and hence failure to induce Mdm-2/HDM2, with consequent accumulation of the mutant p53 protein.[72] Overproduction of Mdm-2/HDM2 is frequently observed in sarcomas; in these cases the normal p53 response to cellular stresses is presumably attenuated through inappropriately efficient p53 degradation.

p53 can also be inactivated by viral oncoproteins, most notably the E6 proteins of human papillomavirus (HPV) types 16 and 18, which are strongly implicated in the genesis of uterine cervical carcinomas.[73] The E6 proteins of these viruses target p53 for ubiquitinylation and degradation via the 26S proteasome, whereas low-risk strains that encode slightly different E6 proteins are not able to transform cells and do not target p53 for degradation.[66] Thus p53 degradation is fundamental to HPV-mediated transformation.

Stabilization and accumulation of p53 can play an important role in apoptosis induced by proteasome inhibitors. Rat-1 and PC12 cells treated with proteasome inhibitors rapidly accumulated p53, as well as the p53 inducible gene products $p21^{WAF1}$ and Mdm-2/HDM2, prior to apoptosis.[61] This could be inhibited by the expression of dominant negative p53, whereas overexpression of wild-type p53 in Rat-1 cells in the absence of proteasome inhibitors was sufficient to induce apoptosis.

In some cases, proteasome inhibitors could nonetheless have therapeutic potential irrespective of tumor p53 genotype or Bcl-2 expression.[74] Proteasome inhibition selectively induced apoptosis in SV40 transformed human fibroblasts but not in the parental untransformed cells.[75] This effect was p53 independent, inhibited by a tetrapeptide caspase inhibitor, and was associated with the accumulation of the CDK inhibitors $p21^{WAF1}$ and $p27^{KIP1}$. In this case the differential induction of apoptosis cannot be explained in terms of differences in proliferation rate, which were similar in the primary and transformed cells. Overexpression of Bcl-2 can prevent apoptosis in response to a variety of stimuli, probably by inhibiting the release of cytochrome *c* from mitochondria.[76] In prostate carcinoma cells stable, overexpression of Bcl-2 failed to protect against cell death induced by proteasome inhibitors, however. A novel dipeptidyl proteasome inhibitor, CEP1612, also induced apoptosis in human Jurkat T cells overexpressing Bcl-2,[75] and similar effects were seen in human breast, tongue and brain tumor cell lines.

Inducible transcription: factors other than p53

In addition to p53, several other transcription factors are regulated by Ub-dependent proteolysis.[77] These include NF-κB, HIF1α, c-Myc, c-Jun and c-Fos, which control a number of important biological functions including the activation of the inflammatory and innate immune responses, apoptosis, vascular development, and the response to oxidative stress.

The NF-κB/Rel family of transcription factors are important ligand-inducible regulators of cell proliferation, inflammation and cellular resistance to apoptosis, and are responsible for the activation of the genes encoding cytokines, chemokines, growth factors, cell adhesion molecules and surface receptors.[78,79] NF-κB-regulated expression of adhesion molecules such as vascular cell adhesion molecule-1 is thought to be involved in tumor metastasis and angiogenesis *in vivo*. Inhibition of NF-κB may limit metastasis by reducing NF-κB-dependent cell adhesion molecule expression as well as making actively dividing cells more sensitive to apoptosis.[80] Members of the NF-κB family are expressed in the majority of cell types, and are active as homodimers and heterodimers.[81] RelA, c-Rel and RelB are expressed in their transcriptionally active forms, while NF-κB1 and NF-κB2 are synthesized as longer precursor molecules of 105 and 100 kDa, respectively. These are processed by the proteasome to generate the transcriptionally active forms of 50 and 52 kDa. Cytosolic NF-κB is bound to the inhibitory IκB protein, which masks the NF-κB nuclear localization sequence, causing cytoplasmic retention. Upon activation by pro-inflammatory stimuli such as tumor necrosis factor (TNF)-α and interleukin (IL)-1, IκB is phosphorylated by the IκB kinase (IKK)[82] and targeted for degradation via the Ub–proteasome pathway. Following IκB degradation and p105/p100 processing, nuclear translocation and DNA binding of the activated NF-κB can occur. Proteasome inhibitors effectively inhibit NF-κB activation *in vitro* and *in vivo*.[35,83] Inhibition of NF-κB has been implicated both in the

induction and in the suppression of apoptosis. For example, overexpression of mutant IκB, which cannot be phosphorylated by IKK and is therefore stable, induces apoptosis, apparently via sequestration of NF-κB. Similarly, activation of NF-κB following exposure to TNF-α, ionizing radiation or daunorubicin inhibited apoptosis.[80] On the other hand, it has recently been suggested that NF-κB is essential for p53-mediated cell death; if this is the case inhibition of NF-κB in tumors that retain functional p53 might be expected to reduce, rather than enhance, therapeutic response.[84]

The c-Myc oncoprotein has the capacity to promote both apoptotic death and, where this death-promoting function is counterbalanced by anti-apoptotic signaling, cell proliferation.[85,86] Studies with Z-LLF-CHO led to the conclusion that c-Myc overexpression was more important than cellular transformation *per se* as a determinant of sensitivity to this proteasome inhibitor.[41] For example, enforced overexpression of c-Myc greatly sensitized Epstein–Barr virus (EBV) immortalized lymphoblastoid cells to Z-LLF-CHO-induced apoptosis. This phenomenon was not attributable to EBV transformation, as Ramos cells, an EBV-negative and c-Myc overexpressing line established from a patient with Burkitt's lymphoma, was also highly sensitive to Z-LLF-CHO. A similar c-Myc-induced sensitization to this inhibitor was observed in a small cell lung cancer line (unpublished observations, cited in Orlowski *et al.*[41]). It is unlikely that c-Myc accumulation can explain all of the observed anti-tumor activity, however, since c-Myc-induced death is thought to be p53-dependent,[87] whereas, as discussed above, proteasome inhibitor-induced death is not.

c-Jun and c-Fos are components of the AP-1 transcription factor complex, and are important regulators of cell proliferation, apoptosis and cellular responses to oxidative stress.[88] As with many inducible transcription factors, the phenotypic change induced by AP-1 activation (and even the polarity of the change) is highly cell type and context dependent. Up-regulation of c-Jun and c-Fos has also been associated with drug resistance.[89–91] The low basal levels of these proteins are achieved in part through Ub-dependent proteolysis, although transcriptional regulation of the factors themselves is also important. The oncogenic activity of v-Jun, a mutant form of c-Jun encoded by an avian sarcoma virus, has been attributed to the comparatively reduced susceptibility of v-Jun to proteasome-mediated degradation.[92] Induction of apoptosis by the peptidyl aldehyde MG132 and other proteasome inhibitors was associated with a steady increase in activity of c-Jun N-terminal kinase, JNK1, which has been implicated in the induction of cell death under a variety of circumstances.[93] Among the known substrates of the proteasome, c-Jun is unusual in that its degradation is not absolutely dependent on polyubiquitinylation. The same was also recently found to apply to the CDK inhibitor $p21^{WAF1}$,[94] but the significance of this apparent bypass of the polyubiquitinylation step is currently unknown.

The unfolded protein response

Aberrantly folded proteins in the secretory pathway are subject to retrograde transport across the ER membrane prior to degradation by 26S proteasomes in the cytoplasm.[95] Inhibition of proteasome function would therefore be expected to result in accumulation of misfolded proteins in the ER. Several agents that induce p53-independent apoptosis do so by perturbing endoplasmic reticulum function and hence engaging an 'unfolded protein response', that involves signaling through the pro-apoptotic transcription factor GADD153/CHOP.[96] If general accumulation of misfolded proteins is a major factor in proteasome inhibitor-induced apoptosis, there may be no need to explain the observed toxicity in terms of accumulation of individual cytosolic or nuclear effectors.

Potential pitfalls

Angiogenesis

HIF1, which is important for the orchestration of cellular responses to hypoxia, is a heterodimeric transcription factor consisting of HIF1α and HIF1β subunits.[97] HIF1 target genes are involved both in increasing oxygen delivery, e.g. by promoting angiogenesis and in allowing metabolic adaptation to reduced oxygen availability. HIF1α protein level and HIF1 transcriptional activity increase dramatically as the cellular oxygen tension decreases. The major basis for this oxygen-responsive regulation is Ub–proteasome-dependent degradation of HIF1α under normoxic conditions.[98] The E3 Ub ligase responsible for oxygen-dependent HIF1α polyubiquitinylation is a multi-protein complex that includes, and depends for its specificity on, the VHL tumor suppressor protein. As a result of *VHL* mutations, HIF1 activity is frequently elevated in renal tumors, which are consequently highly vascular.

Stabilization of HIF1α through inhibition of its Ub-dependent proteolysis could promote expression of HIF1 targets such as vascular endothelial growth factor (VEGF), and hence might be expected to drive tumor angiogenesis. This does not seem to be the case in practice, however. The proteasome inhibitor lactacystin has been shown to reduce plasminogen activator

production in endothelial cells *in vitro* and could therefore inhibit neovascularisation.[99] Furthermore, proteasome inhibitors applied *in vivo* to embryonic chick chorioallantoic membrane, a rapidly expanding tissue, induced apoptosis and vascular regression.[59] Areas devoid of blood flow were found to be due to the induction of apoptosis of endothelial cells and other cells, and to the collapse of capillaries. Despite these encouraging results, it might be valuable to monitor tumor angiogenesis and levels of angiogenic factors in patients participating in phase I trials of proteasome inhibitors.

Drug resistance

Partial loss of function or overexpression of components of the 19S regulatory complex of the proteasome is associated with the acquisition of AP-1-dependent multidrug resistance in yeast and mammalian model systems. For example, transient overexpression of the 19S regulatory subunit POH1 in COS cells conferred P-glycoprotein (P-gp)-independent resistance to taxol, doxorubicin, 7-hydroxy-staurosporine and UV light.[100] On the other hand, a number of studies have suggested that proteasome inhibitors can overcome tumor cell resistance to cytotoxic therapies.

The ATPase P-gp is the prototypic member of a family of membrane-bound transporter proteins potentially involved in multidrug resistance in cancer. Immature core glycosylated P-gp is prevented from reaching the cell surface by proteasome inhibitors;[101] the processing intermediates are retained in the ER in association with the chaperones calnexin and HSC70. It has been suggested that prevention of P-gp maturation could help to overcome drug resistance, but the validity of this suggestion has yet to be established.

In at least some cases resistance to anti-cancer therapies appears to be mediated not by drug pumps, but by resistance to apoptosis. The PML protein has been implicated in apoptotic pathways triggered by Fas, TNF-α, ceramide, IFN, DNA damage and oncogene activation.[102] The PML/RARα (retinoic acid receptor α) fusion protein, generated as a consequence of chromosomal translocation, results in acute promyelocytic leukemia (APL) and acts in a dominant negative fashion to render hemopoietic progenitor cells resistant to Fas-, TNF- and IFN-induced apoptosis. PML is typically found in discrete structures called PML nuclear bodies, which are disrupted by the binding of RARα. Treatment with retinoic acid causes PML to be found in nuclear bodies and induces the degradation of PML/RARα, differentiation of leukemic blasts and disease remission. APL cells resistant to retinoic acid treatment do not express PML/RARα protein, although PML mRNA is detectable at levels comparable to those found in sensitive cells. Proteasome inhibitors have been shown to partially reverse this retinoic acid resistance.[103] The potential for combining retinoic acid and proteasome inhibitor therapies may be limited, however. When used in combination with proteasome inhibitors, levels of retinoic acid required to induce degradation of PML/RARα were associated with toxicity not seen with the same level of retinoic acid alone.[104]

Inhibition of NF-κB activation may promote apoptosis in otherwise resistant cells,[105] specifically those that have lost p53 function.[84] Proteasome inhibitors caused apoptosis *in vitro* in chronic lymphocytic leukemic (CLL) lymphocytes, even if these were resistant to glucocorticoids or nucleoside analogs.[60] This apoptosis was associated with caspase activation and could be blocked by the caspase antagonist ZVADfmk. Proteasome inhibition resulted in mitochondrial dysregulation and the release of cytochrome *c*, changes that were associated with the inhibition of NF-κB. In addition, treatment of some apoptosis-resistant cell lines and primary leukemia cells with subtoxic doses of proteasome inhibitor sensitised these cells to conventional pro-apoptotic agents.[105–107] Similarly, combination of PS-341 with ionizing radiation, cyclophosphamide, 5-fluorouracil, cisplatin, Taxol or adriamycin produced additive tumor growth delays in an EMT-6 murine mammary tumor xenograft model. In contrast, the proteasome inhibitor was not able to overcome the *in vivo* resistance of EMT-6 derivatives previously selected *in vivo* for resistance to cyclophosphamide or cisplatin.[40] The efficacy of proteasome inhibitors against drug-resistant tumors in human subjects has yet to be established.

Neurotoxicity

Lactacystin induced apoptosis in cerebellar granular cells,[108] but in another study proteasome inhibitors were found to prevent apoptosis in sympathetic neurones, suggesting that these drugs might, if anything, be neuroprotective. The *in vivo* distribution of PS341 showed no apparent penetration into the CNS, suggesting that for at least some inhibitors, CNS neurotoxicity might not be a problem *in vivo*.

Prospects

Development of proteasome inhibitors as anti-cancer agents is clearly still in its infancy but, having established that the proteasome is a potentially useful

drug target, the most progress to date has been made using empirical approaches. For example, the selection of PS-341 from a series of dipeptide boronic acid proteasome inhibitors was based on its low mean IC_{50} value when tested against the National Cancer Institute (NCI) 60 cell line panel, although there was a clear relationship between cytotoxicity of this series of inhibitors and proteasome inhibition *in vitro*.[39] This correlation suggests that primary screens of compound libraries could be greatly simplified by the use of *in vitro* assays for proteasome inhibition. Secondary screens against tumor cell line collections could then be used as a measure of bioavailability before activity against xenograft models is tested. In the short term, it seems likely that this general approach will continue to yield novel proteasome inhibitors with increased activity and selectivity.

It has not yet been possible to explain the observed anti-tumor effects of proteasome inhibitors in terms of accumulation of specific proteasome substrates, although there are clearly several good candidates, as discussed above. In the event that accumulation of specific protein(s) is found to explain the anti-cancer activity of proteasome inhibitors, alternative strategies could be used to stabilize these key targets without incurring deleterious effects that might result from general proteasome inhibition. Most of the specificity of degradation is apparently conferred not by the proteasome itself but by the E3 Ub ligases. Substantial research effort is therefore being directed towards targeting specific E3 enzymes using appropriate inhibitors. For example, inhibition of the interaction between p53 and Mdm-2/HDM2 could provide a means of up-regulating functional p53 in a relatively specific manner.[109] Similarly, specific inhibition of IκB degradation was achieved by microinjection of a peptide corresponding to the region of IκB recognized by its E3 Ub ligase.[110] It may be important to give consideration to the state in which proteasome substrates might accumulate following proteasome inhibition. If the proteolysis of polyubiquitinylated substrates is inhibited, it is not clear to what extent these are likely to be biologically active, as the polyubiquitin chains may well perturb function. In the case of p53, even if the polyubiquitinylated protein were re-cycled effectively through the action of Ub–peptide isopeptidase activities, the persistence of Mdm-2/HDM2 would be expected to inhibit the transcriptional activity of p53 and to promote its export from the nucleus.

The genetic heterogeneity of tumors might suggest that accumulation of different substrates underlies the anti-tumor activity of proteasome inhibitors in different tumors. On the other hand, the direct relationship between proteasome inhibition *in vitro* and cytotoxicity against the genetically diverse NCI 60 cell line panel[39] suggests that a more general mechanism is at play. Of all of the candidate mechanisms reviewed above, perhaps the most generally applicable is cell death as a consequence of proteasome inhibitor-induced triggering of the unfolded protein response. The basis for the observed tumor cell specificity remains unexplained, but this is equally true of any number of anti-cancer agents with a broad selectivity for proliferating cells. If the anti-tumor effects so far reported for proteasome inhibitors are translated into successful clinical trials, the increasingly detailed knowledge of the proteasome and its functions might be useful principally in the retrospective explanation of these effects at the molecular level.

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